Szinergia összegző űrlap

(a pályázók közösen ezt az űrlapot töltik ki)

- Adják meg a támogatott szinergia programjuk címét és szakmai fókuszpontját

„Kalmodulin és az ér-reaktivitásban fontos eNOS és MLCK enzimek kölcsönhatásának szabályozása szfingolipid mediátorokkal”

Kutatási projektünk az "1" fókuszponthoz kapcsolódik:

"*Jelátviteli fehérjék szerepe gyulladásos és daganatos megbetegedésekben*"

- Adják meg a szinergia program keretében együttműködő partnerek nevét, tudományos fokozatát, tudományos besorolását, e-mail címét.

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- Csatolják a MedInProt programnak köszönhetően elkészült tudományos közleményeik**,** szakmai megjelenésük bibliográfiai adatait, valamint e dokumentum pdf-ét**.** Minden publikáció esetében fejtsék ki max. 2 mondatban a MedInProt relevanciáját.

1) Juhász T, Ruisanchez É, Harmat V, Kardos J, Kabai M, Benyó Z, Liliom K: Sphingosine inhibits NO-mediated vasorelaxation of mouse thoracic aorta via binding to calmodulin. International Ceramide Conference and Sphingolipid Club Joint Meeting, Cesme, Törökország, 2015. 05. 06-10. Poszter előadás.

A szakterület konferenciáján poszter előadásban ismertettük a MedInProt pályázatunkban a projekt első félévére vállalt és elért eredményeinket.

2) Dancs P, Móré D, Ruisanchez É, Kerék M, Panta C, Caldeira H, Offermanns S, Benyó Z: Signaling pathways of sphingosine-1-phosphate in vascular smooth muscle contraction. International Ceramide Conference and Sphingolipid Club Joint Meeting, Cesme, Törökország, 2015. 05. 06-10. Poszter előadás.

A konferencián poszter előadásban ismertettük a MedInProt pályázatunk megvalósítása során nyert új eredményeinket a szfingozin-1-foszfátnak az értónus és érreaktivitás szabályozásában betöltött szerepéről.

3) Juhász T, Ruisanchez É, Harmat V, Kabai M, Kardos J, Benyó Z, Liliom K, Sphingosine inhibits calmodulin's action. Manuscript under preparation for PNAS.

A MedInProt pályázat támogatásával elért és korábbi eredményeink együttese alapján készülő kézirat a kalmodulin szfingozin általi gátlásáról és annak a kardiovaszkuláris rendszerre potenciálisan kifejtett hatásairól élettani és gyulladásos folyamatokban.

4) Németh T, Ruisanchez É, Hricisák L, Iring A, Horváth B, Smrcka AV, Merkely B, Offermanns S, Benyó Z: Thromboxane receptor signaling in the cardiovascular system. Bioactive Lipids in Cancer, Inflammation, and Related Diseases, 14th International Bioactive Lipids Conference, Budapest, Hungary, July 12-15, 2015. Meghívott előadás.

A konferencián tartott meghívott előadásban a MedInProt pályázatunk megvalósítása során az értónus szabályozásában szerepet játszó tromboxán receptorok intracelluláris jelátviteli folyamataival kapcsolatos új eredményeinket ismertettük.

5) Dancs P, Ruisanchez É, Kerék M, Balogh A, Offermanns S, Tigyi G, Benyó Z: Role of LPA and thromboxane receptors in the lysophosphatidic acid induced vasoconstriction. Bioactive Lipids in Cancer, Inflammation, and Related Diseases, 14th International Bioactive Lipids Conference, Budapest, Hungary, July 12-15, 2015. Poszter előadás.

A poszteren a MedInProt pályázatunk megvalósítása során nyert új eredményeinket ismertettük a lizofoszfolipid LPA értónusra kifejtett hatásait közvetítő receptorokról és intracelluláris jelátviteli folyamatokról.

6) Móré D, Ruisanchez É, Dancs P, Hricisák L, Panta R, Kerék M, Offermanns S, Benyó Z: Mechanism of sphingosine-1-phosphate induced vascular smooth muscle contraction under hyperkalemic conditions. Bioactive Lipids in Cancer, Inflammation, and Related Diseases, 14th International Bioactive Lipids Conference, Budapest, Hungary, July 12-15, 2015. Poszter előadás.

A poszter előadásban a MedInProt pályázatunk megvalósítása során nyert, a szfingozin-1-foszfát hiperkalémiás körülmények között tapasztalható vazokonstriktor hatásának mechanizmusát leíró eredményeinket ismertettük.

7) Juhász T, Ruisanchez É, Harmat V, Kardos J, Kabai M, Benyó Z, Liliom K: Sphingosine inhibits NO-mediated vasorelaxation of mouse thoracic aorta via binding to calmodulin. Bioactive Lipids in Cancer, Inflammation, and Related Diseases, 14th

International Bioactive Lipids Conference, Budapest, Hungary, July 12-15, 2015. Poszter előadás.

A poszter előadásban a MedInProt projekt kezdeti eredményeit ismertettük a szfingozin érrektivitást befolyásoló hatásának mechanizmusáról.

8) Benyó Z, Dancs PT, Ruisanchez É, Kerék M, Balogh A, Nüsing RM, Offermanns S, Tigyi G: Signaling pathways of lysophosphatidic acid induced vasoconstriction. FASEB Science Research Conference on Lisophospholipids and Related Mediators – From Bench to Clinic, Banff, Alberta, Canada, August 23-28, 2015. Felkért előadás.

Az előadásban a MedInProt pályázatunk megvalósítása során a lizofoszfatidsav vazokonstriktor hatásának mechanizmusával kapcsolatban nyert új eredményeinket ismertettük.

- Fejtsék ki pontosan, hogy a kutatási együttműködésük hogyan kapcsolódott az alább megadott MedinProt **fókuszpontok** legalább egyikéhez *(max. 300 szó)*.

"*Jelátviteli fehérjék szerepe gyulladásos és daganatos megbetegedésekben*"

A gazdaságilag fejlett országokban és a fejlődő világban egyaránt súlyos népegészségügyi problémát jelentő szív-érrendszeri elváltozások jelentős részére, közülük különös tekintettel az érelmeszesedésre és a 2-es típusú cukorbetegség érrendszeri szövődményeire, az elmúlt évtized kutatási eredményei alapján mint gyulladásos folyamatok által iniciált és súlyosbított betegségekre tekinthetünk. Ugyanezen időszak élettani és kórélettani kutatásainak eredményeként a szfingolipid mediátorokat ma már a gyulladásos és immun-folyamatok legfontosabb szabályozó molekulái között tartjuk számon (Nature 510: 58-67, 2014). Közülük a szfingozin-1-foszfát elsősorban G-fehérjékhez kapcsolt sejtfelszíni receptorain keresztül fejti ki hatásait, míg a szfingozin, a ceramid és a ceramid-1-foszfát döntően a sejteken belüli jelátviteli fehérjékkel kölcsönhatásba lépve befolyásolja a sejtfunkciókat. A pályázó MTA-TTK munkacsoport által végzett *in vitro* kísérletek eredményei szerint a szfingozin gátolja az erek tónusának és permeabilitásának szabályozásában kiemelt jelentőségű endotheliális nitrogén-monoxid szintetáz (eNOS) enzim aktivitását, mégpedig azáltal, hogy a kalmodulinhoz kötődve gátolja a két fehérje interakcióját, ami az eNOS aktiválódásának alapfeltétele. Az eNOS enzim által termelt nitrogén monoxid (NO) jelátvivő molekula fiziológiás körülmények között vazorelaxáns, valamint gyulladást és thrombus-képződést csökkentő hatású, ezzel szemben bizonyos patológiás körülmények között a túltermelődése gyuladás-keltő, ill. -fokozó hatású (lásd pl. Inflammopharmacology 15: 252-259, 2007). Feltételezzük, hogy a szfingozin és más szfingolipid mediátorok gyulladásos folyamatokban kiváltott érhatásainak egy része a Ca^{2+}-kalmodulin által szabályozott enzimek aktivitásának befolyásolásával jön létre. Irodalmi adatok szerint a szfingozin bizonyos körülmények között hatással van

ezen enzimek közül az eNOS és az érsimaizom kontrakcióját vezérlő miozin könnyűlánc kináz (MLCK) működésére, de a kölcsönhatás mechanizmusa nem ismert. Munkahipotézisünk szerint ezen enzimek szfingolipidek (szfingozin, szfinganin, C_2-ceramid és C_{16}-ceramid) által történő szabályozásának molekuláris alapja a kalmodulin közvetlen gátlása, ezáltal a célenzimek aktivitásának csökkentése.

- Foglalják össze **közérthetően** szinergia programjuk, és közös munkájuk eredményeit *(max. 300 szó).*

1) Megmutattuk, hogy a szfingozin 10 és 50 μM koncentrációban eNOS-függő módon kompetitív jelleggel gátolja az endothelium-függő vazodilatációt, az NO-donor Na^+-nitroprusszid okozta vazorelaxációra azonban nincs hatással. Egér aorta gyűrűkön felvettük a dózis-hatás görbéket, meghatároztuk az EC_{50} értékeket.

2) Megmértük a szfingozin vérnyomásra kifejtett hatását. Altatott egerekben 30 nmol/g (i.a.) szfingozin adása egy kezdeti tranziens hypertenzió után lassan csökkentette az artériás középnyomást és szívfrekvenciát, amely hatás mechanizmusát további mérésekben tisztázzuk.

3) Jellemeztük a kalmodulin-MLCK kötődést és annak szfingozin-általi gátlását in vitro.

4) Tisztáztuk, hogy mind a rövid, mind a hosszú szénláncú ceramidok hatástalanok, nem kötődnek a kalmodulinhoz és annak célenzimjeihez sem.

5) Megmutattuk, hogy a szfinganin és dimetil-szfingozin vegyületek a szfingozin alapvegyülethez hasonló mértékű kalmodulin-kötődéssel és ezen keresztül megvalósuló enzim-gátlással jellemezhetőek.

6) Leírtuk, hogy a szfinganin és dimetil-szfingozin vegyületek gátolják az eNOS-függő vazodilatációt, azonban nem befolyásolják az érsimaizomzat NO-val szembeni válaszkészségét.

7) Felállítottunk egy, az endotél és simaizom sejtek komplex hozzájárulását is figyelembe vevő javított modellt a szfingozin és analógjai érhatásainak mechanizmusára.

- Értékeljék és véleményezzék közös munkájukat (sikereiket, nehézségeiket, illetve azon ötleteiket, javaslataikat, amelyeknek köszönhetően a következő programok hatékonysága javulhat) *(max. 200 szó).*

Előzetes eredményeink szerint a szfingozin gátolja az erek tónusának és permeabilitásának szabályozásában kiemelt jelentőségű endotheliális nitrogén-monoxid szintetáz (eNOS) enzim aktivitását, mégpedig azáltal, hogy a kalmodulinhoz

(CaM) kötődve gátolja a két fehérje interakcióját, ami az eNOS aktiválódásának alapfeltétele. Munkahipotézisünk szerint a szfingozin és más szfingolipid mediátorok gyulladásos folyamatokban kiváltott érhatásainak egy része a Ca^{2+}-CaM által szabályozott enzimek aktivitásának közvetlen befolyásolásával jön létre. Ezen enzimek közül az eNOS és az érsimaizom kontrakcióját vezérlő miozin könnyűlánc kináz (MLCK) működésének szfingolipidek (szfingozin, szfinganin, C_2-ceramid és C_{16}-ceramid) által történő szabályozásának molekuláris mechanizmusait vizsgáltuk a MedInProt projekt keretében. Megmutattuk, hogy a szfingozin, szfinganin és dimetil-szfingozin ex vivo aorta-szegmenseken gátolja az eNOS aktivitását, illetve a szfingozin és analógjai gátolják az MLCK enzimet, míg a vizsgált ceramidok hatástalanok. A szinergia-program keretében elért eredményeink erőssége, hogy in vitro vizsgált molekuláris interakciók biológiai funkciókra kifejtett hatásait tudtuk demonstrálni, így reményeink szerint lényegesen magasabb színvonalú szakfolyóiratban közölhetők, és ami ennél is fontosabb, hogy szélesebb kutatói közösség érdeklődésére tarthatnak számot és további kollaborációs kutatások kiindulópontját jelenthetik.

- Szabadon fogalmazzák meg a MedInProt kapcsán támogató és/vagy kritikus észrevételeiket. *(max. 200 szó)*

A MedInProt programot a hazai kutatásfinanszírozási rendszerben úttörő kezdeményezésnek tartjuk, mely a szinergizmusok kiaknázása révén a befektetett forrásokat rendkívül hatékonyan képes kutatási eredményekké konvertálni. Ez egyben a hazai tudományos élet kritikája is, mert azt jelzi, hogy az országon belüli tudományos együttműködésekben rejlő lehetőségek messze nincsenek kiaknázva. Egyaránt vonatkozik ez a megállapítás a szakértelem, valamint a kutatási infrastruktúra kutatóhelyek és kutatócsoportok közötti megosztására. A MedInProt sikere felhívja a figyelmet erre a hiányosságra, egyúttal a fejlődése során szerzett tapasztalatok bizonyára felhasználhatóak lesznek majd a tudománypolitika számára a probléma megoldási lehetőségeinek keresésében.

A program mozgósító ereje jelentős részben annak köszönhető, hogy a résztvevőkre háruló bürokratikus terhek hazai viszonylatban viszonylag alacsonyak, a pályázati adatbekérés jól fókuszált, célirányos, a számonkérési rendszer átlátható. A döntési rendszer jól szervezett és gyors. Nyilván ezek az előnyök jelentős részben annak is köszönhetők, hogy kis volumenű pályázatokról van szó, de üdvös lenne, ha ennek a sikeres modellnek egyes elemei átültethetők lennének más, nagyobb, jelenleg túlbürokratizált pályázati rendszerekre is.

A szinergizmus szakmai fókuszpontjai, kiemelt kutatási témák:

1) *Jelátviteli fehérjék szerepe gyulladásos és daganatos megbetegedésekben,*

2) *NMR és MRI adta lehetőségek a fehérjék feltekeredésével kapcsolatos betegségek molekuláris hátterének megértésében,*

3) *Szabályozó fehérjék szerepe az öregedési folyamat(ok)ban,*

4) *Alkalmas nanorendszerek fejlesztése peptid- és fehérjealapú hatóanyagok stabilitásának és felszívódásának fokozása érdekében.*

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Sphingosine inhibits NO-mediated vasorelaxation of mouse thoracic aorta via binding to calmodulin

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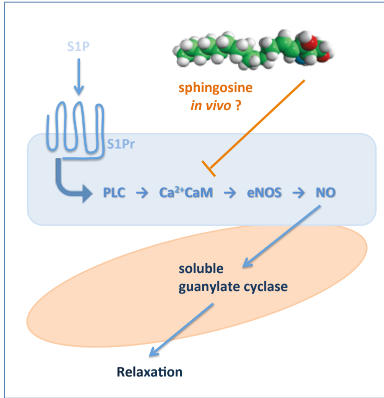
[2]*Instutite of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest;*
[3]*Laboratory of Structural Chemistry and Biology, Institute of Chemistry, and* [4]*Department of Biochemistry, Institute of Biology, Eötvös Loránd University, Budapest*

Hypothesis



We hypothesize that the lipid mediator sphingosine (Sph) can exert its vasoactive effects via its ability to inhibit the calcium-saturated calmodulin (Ca^{2+}CaM), and thereby preventing activation of the Ca^{2+}CaM-dependent endothelial nitric oxide (NO) synthase (eNOS), and NO-dependent vasorelaxation.
S1P: sphingosine-1-phosphat; S1Pr: S1P receptor;
PLC: phospholipase C

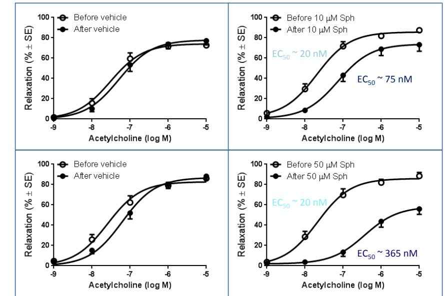
OBJECTIVE
Calmodulin-dependent endothelial nitric oxide synthase (eNOS) is a main determinant of the vascular tone and permeability. The sphingolipid mediator sphingosine (Sph) is suggested to have vasoactive properties. We investigated the effect and mechanism of action of sphingosine on the NO-dependent vasorelaxation.

METHODS
To test the ability of Sph to alter eNOS activity in intact vessels, myography experiments were performed on mouse thoracic aorta *ex vivo*. We compared the acetylcholine-induced eNOS-mediated relaxation before and during incubation of the vessels with Sph. The binding of Sph to calmodulin and its effect on calmodulin's function *in vitro* were characterized by fluorescence spectroscopy, isothermal titration calorimetry, and crystallography.

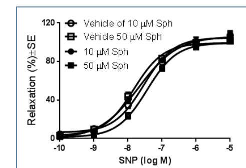
RESULTS
Treatment of the intact vessels with Sph induced significant rightward shift of the acetylcholine dose-response curve. Sphingosine exhibited no significant effect when vasodilation was elicited by the direct NO-donor sodium nitroprusside. We characterized in vitro the inhibition by Sph of calmodulin-dependent activity of eNOS, phosphodiesterase, and calcineurin. Calmodulin was found to bind Sph *in vitro* with low nanomolar affinity when the lipid was clustered. The crystal structure of their complex showed few lipid molecules wrapped around by the protein in its inhibitory conformation.

CONCLUSIONS
NO under physiologic conditions is a vasorelaxant and attenuates inflammation, while its increased production leads to the initiation and progression of inflammation. We have shown here that the vascular effects of sphingosine in inflammation might be due to its influence on the activity of Ca^{2+}-calmodulin-dependent eNOS enzyme *via* binding to calmodulin thereby preventing eNOS activation.

Ex vivo eNOS functional assay

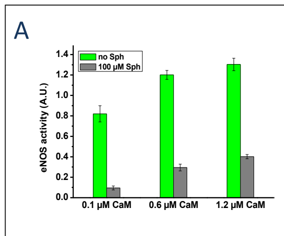


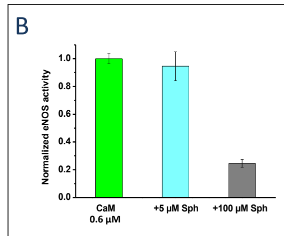
Treatment of the intact mouse thoracic aorta vessels with Sph induced significant rightward shift of the acetylcholine dose-response curve, while Sph also showed a tendency to decrease the maximal relaxing effect. Incubation of the vessels with the appropriate vehicle resulted in no significant effect.



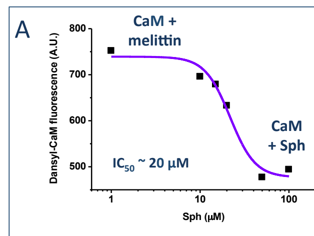
No significant effect of Sph on the SNP-mediated vasorelaxation could be detected, which indicated that the inhibitory effect of Sph is not due to the decreased NO sensitivity of the vascular smooth muscle.

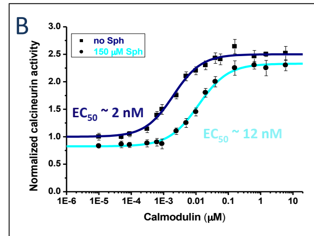
In vitro functional assays





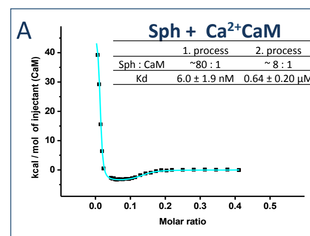
A) NOS was activated by various amounts of CaM, which was inhited by 100 μM Sph.
B) Sph inhibited the CaM-induced eNOS activity concentration-dependently: the effect is observed at concentrations above the CMC value (~ 15 μM).



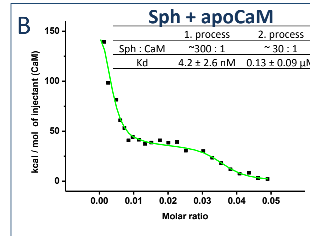


A) Sph displaces the model CaM binding domain melittin from CaM, the effect is measured at about the CMC value of Sph.
B) CaM activates the phosphatase calcineurin, and Sph causes a rightward shift of the curve indicating a competitive inhibition of the CaM-induced activation by Sph.

Sph binding to CaM: Stoichiometry and affinity



	1. process	2. process
Sph : CaM	~80 : 1	~8 : 1
Kd	6.0 ± 1.9 nM	0.64 ± 0.20 μM

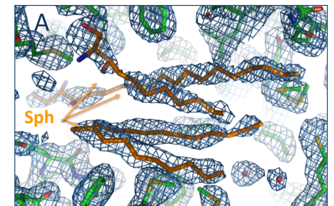


	1. process	2. process
Sph : CaM	~300 : 1	~30 : 1
Kd	4.2 ± 2.6 nM	0.13 ± 0.09 μM

For both Ca^{2+}CaM and apoCaM, two binding processes were detected utilizing ITC:
1. binding of CaM to micelle surface
2. disintegration of lipid micelles

2A) binding of Sph monomers into the CaM → see the Ca^{2+}CaM - Sph crystal structure
2B) formation of mixed lipid-protein „micelles" (apoCaM + Sph)

The crystal structure of the Ca^{2+}CaM - Sph complex



A-B) In the Ca^{2+}CaM - Sph complex, four Sph monomers bound in the central channel of CaM can be identified.
A) The lipids showed lower electron density so that not all parts of the Sph molecules could be determined.

Acknowledgement

K 82092 to KL, K 112964 to ZB, PD 104344 to TJ

Methods:

Ex vivo eNOS assay: the preparation of vessels and the myography experiments were performed as published in Horvath et al, J CARDIOVASC PHARMACOL, 2005. 45(3): P. 225-31; *In vitro eNOS assay:* eNOS (Sigma) activity was measured using the Ultrasensitive Colorimetric NOS Assay kit and the NOS cofactor mix (Oxford Biomedical Research) according the manufacturer's instructions; *Melittin assay*: melittin binding was measured fluorometrically with 0.2 μM dansyl-labelled CaM, and 0.4 μM melittin in the presence of 0-200 μM Sph; *Calcineurin assay*: calcineurin (Promega) activity was followed according to the manufacturers instruction (or see: Kovacs and Liliom, BIOCHEM J, 2008. 410(2): P. 427-37; *Isothermal titration calorimetry:* 10 mM HEPES, 100 mM KCl, pH 7.2 buffer + 100 μM EGTA or 5 mM $CaCl_2$, 25 °C, 200 μM Sph in the cell was titrated with 40-400 μM CaM. *Crystallization:* hanging drop method; rezervoir: 50 mM Na-cacodilate, 10-10 mM $CaCl_2$ and $MgCl_2$, 15-25% PEG8000, pH 4,5-5,5; drop: 2 μl rezervoir, 2 μl 0.7 mM Ca^{2+}CaM + 1 μl 10 mM Sph in methanol.

P58: SIGNALING PATHWAYS OF SPHINGOSINE-1-PHOSPHATE IN VASCULAR SMOOTH MUSCLE CONTRACTION

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Objectives: We aimed to examine the direct effect of sphingosine-1-phosphate (S1P) on vascular smooth muscle (VSM) contractility and to elucidate the underlying signaling pathways.

Methods: Isometric tension of endothelium-denuded thoracic aorta segments isolated from male wild type (WT) and knockout (KO) mice deficient in S1P2, S1P3 receptor or Gα12/13 was measured in myographs. Vasoactive effect of 10 µM S1P was detected at physiological (4 mM) and elevated (6-124 mM) extracellular K+-concentration [K+]e.

Results: At physiological [K+]e S1P had negligible vasoactive effect. Slightly increased [K+]e (6 mM) failed to influence the vascular tone by itself, but addition of S1P induced marked vasoconstriction that was further intensified if [K+]e was increased to 8 and 10 mM. At higher [K+]e (20-80 mM), K+ increased the vascular tone progressively and attenuated the additional vasoconstriction by S1P. Further experiments on the signaling pathways were performed with 8 mM [K+]e, which enhanced the action of S1P markedly without significantly influencing the resting vascular tone by itself. The vasoconstrictor effect of S1P disappeared in S1P2-KO and Gα12/13-KO vessels, whereas remained unchanged in S1P3-KO mice. S1P-induced vasoconstriction was also abolished by the Rho-kinase inhibitor Y-27632 (10 µM) in WT vessels.

Conclusion: S1P induces vasoconstriction in case of moderately increased [K+]e. The effect of S1P is mediated by S1P2-receptor, Gα12/13, and Rho-kinase. This phenomenon may lead to increased vascular tone under conditions of systemic or local elevation of [K+]e surrounding the VSM, like in hyperkalemia or tissue ischemia. Grant support: OTKA K-101775 and MedInProt Protein Science Research Synergy Program.

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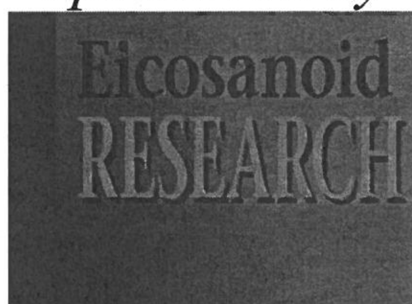

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Thromboxane receptor signaling in the cardiovascular system

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Introduction. Thromboxane A_2 (TXA_2) is a key mediator of diverse physiological and pathophysiological functions in the vascular system and it may induce sustained vascular smooth muscle (VSM) contraction via stimulation of TXA_2 prostanoid receptors (TPs). We aimed to identify the heterotrimeric G-proteins and their downstream signaling pathways mediating the vasoactive effects of TP activation.

Materials and methods. Isometric tension recording was performed in thoracic aortic segments (TAs) isolated from wild-type (WT) or phospholipase Cε knock out (PLCε-KO) mice as well as from mice deficient in $G\alpha_{q/11}$- or $G\alpha_{12/13}$-proteins in the smooth muscle (SM-$G_{q/11}$-KO and SM-$G_{12/13}$-KO) (1). Intracellular calcium levels $[Ca^{2+}]_i$ were assessed by ratiometric measurement of Fura-2AM fluorenscence in the VSM.

Results. The moderate vasoconstriction induced by low concentrations (1-30 nM) of the TP agonist U-46619 in WT TAs was sensitive to the Rho-kinase (ROCK) inhibitor Y-27632, and it remained unaltered in SM-$G_{q/11}$-KO but disappeared in SM-$G_{12/13}$-KO vessels. Weak TP stimulation with 10 nM U-46619 markedly aggravated the vasoconstrictor actions of the α_1-adrenoreceptor agonist phenylephrine or that of serotonin in WT but not in SM-$G_{12/13}$-KO vessels.

In contrast, higher concentrations of U-46619 induced strong and nitric oxide resistant vasoconstriction, which was only partially reversed by either $G\alpha_{q/11}$- or $G\alpha_{12/13}$-deletion, indicating the involvement of both pathways in mediating the effect. Surprisingly, SM-$G_{q/11}$-KO VSM, which was completely unresponsive to phenylephrine, showed a significant $[Ca^{2+}]_i$ elevation for U-46619, which persisted in Ca^{2+}-free medium, and was unaffected by Y-27632, but was almost completely abolished by the RhoA inhibitor TAT-C3. TAs of mice deficient in the RhoA-sensitive PLCε (2) showed attenuated vasoconstriction and decreased VSM Ca^{2+}-signal upon administration of U-46619, as compared to WT controls. In accordance, the hypertensive effect of U-46619 but not that of norepinephrine was markedly reduced in PLCε-KO mice.

Conclusions. Our results indicate that weak activation of TPs appears to increase the vascular tone and reactivity exclusively via $G_{12/13}$-RhoA-ROCK signaling, whereas strong TP stimulation activates both the $G_{q/11}$- and the $G_{12/13}$ pathways and induces a sustained intracellular Ca^{2+} release via a RhoA-dependent, but ROCK-independent activation of PLCε. This unique signaling mechanism is likely to be responsible for spastic vasoconstrictions induced by overactivation of TPs under pathophysiological conditions.

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1. Nat Med. 14: 64-68, 2008; doi: 10.1038/nm1666
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94 Role of LPA and thromboxane receptors in the lysophosphatidic acid induced vasoconstriction

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Introduction. Lysophosphatidic acid (LPA) is a bioactive lipid mediator with significant physiological effects in immunbiology and hemostasis. We have recently reported that in intact vessels LPA induces endothelial NO-mediated vasorelaxation (1). On the contrary, if the endothelium was removed, LPA caused vasoconstriction. Here we report on our findings on the signaling pathways mediating this effect of LPA.

Materials and methods. Aortic segments were isolated from wild type (WT) and knockout (KO) mice deficient in LPA_1 or LPA_2 receptors, cyclooxygenase-1 (COX1) or thromboxane receptors (TP). Vessels of mice subjected to smooth muscle-specific deletion of $G\alpha_q$ and $G\alpha_{11}$ proteins (SM-$G_{q/11}$-KO) (2) were also tested. Isometric tension of endothelium-denuded vessels was measured via myography. Expression of LPA receptors in vascular smooth muscle (VSM) was analyzed by qPCR. Thromboxane A_2 (TXA_2) release of murine aorta was measured by TXB_2 ELISA.

Results. In the absence of endothelium LPA elicited vasoconstriction. PCR analysis revealed the presence of mRNA encoding type 1, 2, 4 and 6 LPA receptors in VSM. The LPA_{1-3} receptor agonist VPC31143 mimicked LPA-induced contraction, whereas the $LPA_{1/3}$ receptor antagonist Ki16425 inhibited the effects of both LPA and VPC31143. Lack of LPA_1 but not that of LPA_2 abolished both LPA- and VPC31143-induced contraction.

Genetic deletion of $G_{q/11}$ signaling and pharmacological inhibition of $G_{i/o}$ proteins by pertussis toxin (PTX) inhibited the vasoconstrictor effect of VPC31143. PTX treatment of SM-$G_{q/11}$-KO mice caused a further decrease in the ex vivo vascular reactivity to VPC31143 indicating the simultaneous involvement of $G_{i/o}$ and $G_{q/11}$ pathways in LPA_1-mediated vasoconstriction.

Because $G_{i/o}$ signaling is often coupled to prostanoid production, the potential involvement of autocrine/paracrine TXA_2 release was tested. Absence of either COX1 or TP receptors diminished LPA- and VPC31143-evoked contraction. Furthermore, VPC31143 markedly increased TXA_2 production in WT and TP-KO but not in COX1-KO vessels.

Conclusions. Our results indicate that in the absence of endothelium - contrary to intact vessels - LPA induces vasoconstriction which is mediated by LPA_1 receptors. Downstream signaling involves both the $G_{q/11}$ and $G_{i/o}$ pathways and COX1-mediated autocrine/paracrine release of TXA_2 leading to activation of TP receptors. Our study may contribute to a better understanding of the Janus-faced role of LPA in the regulation of vascular tone under physiological conditions and in pathophysiological states associated with endothelial dysfunction.

1. FASEB J. 28: 880-890, 2014; doi:10.1096/fj.13-234997
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95 Lipid Second Messengers Formation in Blood Mononuclear Cells in Acute Leukemia

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Recent studies suggest a potential impact of changes in different subpopulation of immune cells on hematological malignancies resulted in the alteration of balance between pro- and anti-tumor immunity. It was hypothesized by us earlier that in such intricate system diseases, as human tumors, alterations in plasma membrane lipid homeostasis in the peripheral blood crude mononuclear cells (MNC) may possibly represent some information useful for cancer detection and estimation. In this study we investigated regularities of different lipid second messengers (LSM) formation in [14C]arachidonic acid (AA)-prelabelled intact MNCs at different time points (5, 10, 30 and 60 sec) following T cell costimulation by anti-CD3 and anti-CD28 antibodies in acute lymphoblastic and myeloblastic forms of leukemia.

The data obtained provide evidence for reproducible defects in the dynamic processes of AA-LSMs generation/utilization by stimulated MNCs in acute leukemia studied. Particularly, the reliable generation of 1,2-diacylglycerol observed in norm at the early (5 sec) step of cell stimulation was completely depressed in two forms of acute leukemia. The elevated levels of AA- lysophosphatidylcholine and free AA simultaneously indicated deregulation of lipid homeostasis in crude MNCs of leukemia patients compared to the norm. Importantly, identical alterations were revealed also in chronic lymphocytic leukemia and some solid tumors, at the early and relatively sustained stages of cell stimulation.

We conclude that identical disturbances revealed in the mechanisms of LSMs generation in peripheral blood crude MNCs reflect the availability and stage of cancer development. These experimental data can be used for early detection and assessment of malignancy.

The author would like to express his gratitude to colleagues from the Laboratory of Regulation of Cellular Activity (Institute of Molecular Biology NAS RA) as well as to all healthy and diseased subjects involved in this study for assistance and support.

152 Mechanism of sphingosine-1-phosphate induced vascular smooth muscle contraction under hyperkalemic conditions

Dorottya Móré[1], Éva Ruisanchez[1], PETER DANCS[1], László Hricisák[1], Cecília Rita Panta[1], Margit Kerék[1], Stefan Offermanns[2], Zoltán Benyó[1]

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Introduction. Sphingosine-1-phosphate (S1P) is a lysophospholipid mediator that is known to affect diverse vascular functions such as development, remodeling and permeability acting on its G-protein coupled receptors ($S1P_{1-5}$). Its role in the regulation of the vascular tone remains controversial. We aimed to examine the direct effect of S1P and the signaling mechanisms involved on vascular smooth muscle (VSM) contractility under physiological conditions and after increasing the VSM excitability by increasing extracellular K^+ concentration $[K^+]_e$.

Methods. Segments of the thoracic aorta were isolated from adult male wild type (WT), as well as $S1P_2$, $S1P_3$ receptor, and $G\alpha_{12/13}$ knockout (KO) mice and their isometric tension was measured in myographs after removal of the endothelium. Vasoactive effect of 10 μM S1P was detected at physiological (4 mM) as well as at elevated (6-8 mM) $[K^+]_e$. Changes of the vascular tension are expressed as mean±SEM percentage of a reference contraction induced by 124 mM K^+.

Results. At physiological $[K^+]_e$ S1P slightly increased the vascular tone (21±5%), whereas sphingosine had no measurable effect. Slightly increased $[K^+]_e$ (6 mM) failed to influence the vascular tone by itself, but addition of S1P induced marked vasoconstriction (39±7%) that was further increased when $[K^+]_e$ was elevated to 8 mM (54±6%).

Further experiments on the signaling of vascular smooth muscle contraction were performed with 8 mM $[K^+]_e$, since it intensified the vasoactive action of S1P without having a significant influence on the resting vascular tone by itself. The vasoconstrictor effect of S1P was diminished in $S1P_2$-KO (11±3%) and $G\alpha_{12/13}$-KO (8±4%) vessels, whereas it remained unchanged in $S1P_3$-KO mice (44±7%). S1P-induced vasoconstriction was also strongly decreased by the Rho-kinase inhibitor Y-27632 (10 μM) in WT vessels (17±1%).

Conclusion. S1P significantly elevates the VSM tone in isolated mouse aorta under moderately increased K^+-concentration. This effect is mediated by the $S1P_2$-receptor, $G\alpha_{12/13}$ proteins, and the activation of Rho-kinase. This phenomenon may contribute to the pathological increase of the vascular tone under conditions of systemic hyperkalemia or during local elevation of perivascular $[K^+]_e$ (eg. due to ischemic tissue infarction).

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153 Inhibition of 12-Lipoxygenase activity in an esophageal cancer cell line decreases proliferation and angiogenic gene expression while its metabolite 12(S)HETE is mitogenic in vitro and increased in cancer serum samples

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Introduction: Lipoxygenase activity has been shown to be increased in a number of cancers types and to have an involvement in tumourogenesis and angiogenesis. There is little known on the role of these enzymes in esophageal cancer, a malignancy that is reportedly the 8th most common cause of cancer globally. In this study we aim to investigate the role of 5-lipoxygenase (5LO) and platelet-type 12-Lipoxygenase (12LO) in oesophageal cancer in vitro and in vivo.

Methods: Esophageal adenocarcinoma cell line (OE33) were investigated for the expression of 5LO, 5LO associated protein (FLAP) and 12LO and 15-lipoxygenase (15LO), in addition to metabolite receptors BLT1, BLT2 and GPCR31 by qPCR. The effect of novel and commercial 5 and 12LO inhibitors on proliferation of OE33s by BrdU was investigated. The effect of inhibitor treatments to OE33s angiogenic gene profile was conducted using gene arrays. 12LO was over-expressed in OE33s by reverse transfection and proliferative effects were monitored using real-time xCELLigence. Additionally serum levels of LTB4 and 12(S)HETE were measured by ELISA in cancer patients samples (N=68), collected pre-surgery and compared to a small cohort of non-cancer control samples.

Results: OE33s were shown to constituently express high 5LO, 12LO and FLAP but low levels of 15LO. OE33s expressed BLT1 however BLT2 and GPCR31 were not detected. While the FDA approved 5LO inhibitor Zileuton did not affect cell proliferation of OE33s, novel 5LO inhibitor, NIH4, showed a dose response effect (IC25= 10uM). A novel 12LO inhibitor NIH13 was shown to have superior anti-proliferative effects compared to commercially available 12LO inhibitor Baicalein, showing an IC50 of 1uM ($p<0.01$). This compound was not shown to induce marked apoptosis in OE33s at 5 and 10uM doses at 24-72hr, suggesting another mechanism is responsible for the anti-proliferative effects. Neuropilin-1 (NRP1) and VEGFB were found to be significantly down regulated by 5uM NIH13 treatment ($P<0.05$) while VEGFA was up regulated (P=0.0515). Over expression of 12LO in OE33s resulted in a significant increase in cell proliferation compared to the vector control. Addition of 12(S)HETE displayed mitogenic effects in 12LO over expressing cells and was able to suppress the effect of NIH13 treatment. There was no difference in mean LTB4 levels between the cancer and non-cancer serums. Serum 12(S)HETE was higher in 75% of cancer patients compared to baseline levels of a small non-cancer cohort ($p<0.05$) and 12(S)HETE levels were associated with recurrence-free survival ($p<0.01$).

Conclusion: The study demonstrates the potential of 12LO activity as biomarker of disease and highlights the potential for a novel class of LO inhibitors as anti-cancer drugs for esophageal cancer. Understanding the molecular mechanisms underlying the anti-tumor effect of these inhibitors may prove useful in the treatment of cancer, either alone or in combination with conventional therapies.

We would like to sincerely thank Prof. Kenneth Honn for providing the ALOX12 over expression vector for this work. We also acknowledge the support of the Irish Research Council in funding this work through an EMBARK initiative PhD scholarship.

Sphingosine inhibits NO-mediated vasorelaxation of mouse thoracic aorta via binding to calmodulin

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OBJECTIVE

Calmodulin-dependent endothelial nitric oxide synthase (eNOS) is a main determinant of the vascular tone and permeability. The sphingolipid mediator sphingosine is suggested to have vasoactive properties. We investigated the effect and mechanism of action of sphingosine on the NO-dependent vasorelaxation.

METHODS

To test the ability of sphingosine to alter eNOS activity in intact vessels, myography experiments were performed on mouse thoracic aorta ex vivo. We compared the acetylcholine-induced eNOS-mediated relaxation before and during incubation of the vessels with sphingosine. The binding of sphingosine to calmodulin and its effect on calmodulin's function in vitro were characterized by fluorescence spectroscopy, isothermal titration calorimetry, and crystallography.

RESULTS

Treatment of the intact vessels with sphingosine induced significant rightward shift of the acetylcholine dose-response curve. Sphingosine exhibited no significant effect when vasodilation was elicited by the direct NO-donor sodium nitroprusside. We characterized in vitro the inhibition by sphingosine of calmodulin-dependent activity of eNOS, phosphodiesterase, and calcineurin. Calmodulin was found to bind sphingosine in vitro with low nanomolar affinity when the lipid was clustered. The crystal structure of their complex showed few lipid molecules wrapped around by the protein in its inhibitory conformation.

CONCLUSIONS

NO under physiologic conditions is a vasorelaxant and attenuates inflammation, while its increased production leads to the initiation and progression of inflammation. We have shown here, that the vascular effects of sphingosine in inflammation might be due to its influence on the activity of Ca^{2+}-calmodulin-dependent eNOS enzyme via binding to calmodulin thereby preventing eNOS activation.

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LYSOPHOSPHOLIPIDS AND RELATED MEDIATORS – FROM BENCH TO CLINIC

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Lysophospholipid Mediators in the Pathophysiology of Vascular Function

Poster #27

Signaling pathways of lysophosphatidic acid induced vasoconstriction

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Introduction. Lysophosphatidic acid (LPA) has recently been reported to induce vasorelaxation in the presence, whereas vasoconstriction in the absence of intact endothelium (1). The vasorelaxation is mediated by LPA_1 receptors, phospholipase C (PLC) and nitric oxide (NO). Here we aimed to analyze the signaling pathways of the vasoconstrictor action of LPA in the vascular smooth muscle (VSM).

Materials and methods. Aortic segments were isolated from wild type (WT) and knockout (KO) mice deficient in LPA_1 or LPA_2 receptors, cyclooxygenase-1 (COX1) or thromboxane receptors (TP). Vessels of mice subjected to SM-specific deletion of $G\alpha_q$ and $G\alpha_{11}$ proteins (SM-$G_{q/11}$-KO) (2) were also tested. Isometric tension of the de-endothelialized vascular segments was measured via myography. Expression of LPA receptors in aortic VSM was analyzed by qPCR. Thromboxane A_2 (TXA_2) release from the aorta was measured by TXB_2 ELISA.

Results. Presence of mRNA encoding type 1, 2, 4 and 6 LPA receptors was detected in the VSM and LPA induced dose-dependent vasoconstriction in aortic rings, which effect was reproduced by the $LPA_{1\text{-}3}$ receptor agonist VPC31143. The $LPA_{1/3}$ receptor antagonist Ki16425 inhibited the effects of both LPA and VPC31143. Lack of LPA_1 but not that of LPA_2 abolished both LPA- and VPC31143-induced vasoconstrictions. Both the genetic deletion of $G\alpha_{q/11}$ proteins and pharmacological inhibition of $G_{i/o}$ mediated signaling by pertussis toxin (PTX) inhibited the vasoconstrictor effect of VPC31143. PTX treatment of SM-$G_{q/11}$-KO mice induced a further reduction in the ex vivo vascular reactivity to VPC31143 indicating the simultaneous involvement of $G_{i/o}$ and $G_{q/11}$ pathways in LPA_1-mediated vasoconstriction. Because $G_{i/o}$ signaling is often coupled to phospholipase A_2 activation and consequent prostanoid production by COX1, the potential involvement of autocrine/paracrine TXA_2 release in the LPA_1-mediated vasoconstriction was tested. Vessels lacking COX1 or TP receptors showed substantially (by 70-80%) diminished vasoconstrictor responses to LPA and VPC31143. In accordance, VPC31143 increased similar TXA_2 production in WT and in TP-KO but not in COX1-KO vessels.

Conclusions. In the absence of endothelium - contrary to intact vessels - LPA induces vasoconstriction which is mediated by LPA_1 receptors. Downstream signaling pathways involve both $G_{q/11}$ and $G_{i/o}$ proteins as well as COX1-mediated autocrine/paracrine release of TXA_2 resulting in activation of TP receptors. Our present and previous findings together indicate that activation of endothelial and VSM LPA_1 receptors results in opposite changes of the vascular tone and the summated effect depends on the functional integrity of the endothelium.

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